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PROCESS FOR FOLDING OF PROTEINS LIKE RECOMBINANT HIRUDIN OR EPIDERMAL GROWTH FACTOR

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Abstract

The invention relates to a process for the preparation of a biologically active and correctly folded protein in the presence of a denaturing agent like urea or guanidine hydrochloride and the separation of the correctly folded protein therefrom directly. This process can be applied, for example, in the renaturation of recombinant proteins like hirudin or epidermal growth factor.

Description

Process for folding of proteins like recombinant hirudin or epidermal growth factor.

The invention relates to a process for the preparation of a biologically active and correctly folded protein in the presence of a denaturing agent.

Correct folding of genetically engineered proteins is a major issue in protein science. The problem is particularly acute, e.g., in the case of disulfide-containing proteins, which often need to be denatured and refolded to become active. Disulfide formation is an event of post-translational modification. It follows the pathway of protein folding and usually occurs faithfully in vivo. However, in the in vitro renaturation experiments, many proteins are recovered in poor yields and some do not fold into native conformation at all. To overcome this problem, a number of compounds, such as the mixture of reduced I oxidized gluta thione (Creighton, Methods Enzymol. 131 (1986), 83-106) and protein disulfide isomerase (PDI) (Morin, J. E. & Dixon, J. E. Methods Enzymol. 113 (1985), 541-547) have been routinely used to promote the formation of disulfides. But despite their widespread use, the precise mechanism of their function remains undefined and their applications have been conducted in a manner of trial and error.

A number of publications have appeared which report refolding attempts for individual proteins produced in bacterial hosts, or which are otherwise in a denatured or non-native form. Formation of a dimeric, biologically active human colony stimulating factor (CSF-1) after expression in E. coli is described in WO 88/8003 and by Halenbeck et al.

Biotechnology, 7 (1989), 710-715. The procedures described involve the steps of initial solubilization of CSF-1 monomers isolated from inclusion bodies under reducing conditions in a chaotropic environment comprising urea or guanidine hydrochloride, refolding which is achieved by stepwise dilution of the chaotropic agents, and final oxidation of the refolded molecule in the presence of a redox-system. In WO 88/8849 a process for recovering recombinant interleukin-2 (IL-2) is disclosed, characterized in that IL-2 isolated from refractile bodies is denatured under reducing conditions with 6M guanidine hydrochloride, the soluble IL-2 is oxidized by a controlled oxidation in the presence of Cu2+ ions, and the oxidized IL-2 refolded by reducing the concentration of the denaturant in the solution.

Interleukin-2 and interferon-ss have been refolded using SDS for solubilization and Cu2+ ions as oxidation promoters of the fully reduced protein (US-A-4572798). The process for isolating recombinant refractile proteins as described in US-A-4620948 involves strong denaturing agents to solubiiize the proteins, reducing conditions to facilitate correct folding and denaturant replacement in presence of air or other oxidizing agents to reform the disulfide bonds. A method for renaturing unfolded proteins including cytochrome c, ovalbumin and trypsin inhibitor by reversibly binding of the denatured protein to a solid matrix and stepwise renaturing it by diluting the denaturant is disclosed in WO 86/5809.

The foregoing references are merely representative of a huge amount of literature dealing with the refolding of non-native proteins derived from different sources. The man skilled in the art on the other hand knows that the success of refolding experiments cannot be predicted. Unsuccessful experiments are usually not reported. There is no certainty that anyone of the reported refolding conditions would work at all with a given denatured protein containing several cysteine residues and therefore, a number of intramolecular disulfide bonds, which are required for activity.

Guanidine hydrochloride (GdmCl) and urea are the best known denaturants for unfolding and inactivating proteins. Although the mechanism of their actions remains to be fully understood, it is generally evident that they disrupt the non-covalent interactions which stabilize the native conformation. The detrimental effect of GdmCl and urea has also been illustrated during the folding of proteins, which usually leads to the formation of inactive, scrambled species (Haber and Anfinsen, J. Biol. Chem. (1962), 237, 1839-1844; Weissman and Kim Science (1991), 253, 1386-1393). On the other hand, denaturants are potent agents for solubilizing intractable proteins, such as immunoglobulins and membrane components etc. For example, recombinant proteins expressed in an Eschedchia coli system often face this problem of protein solubility. These proteins are frequently found in insoluble inclusion bodies and require solubilization by strong denaturant as the essential step to refold and generate the active conformation.

Surprisingly it has now been found that in the presence of denaturant and under equilibrium conditions correct folded protein is formed and can be isolated therefrom directly. This finding greatly facilitate the preparation of recombinant proteins as these proteins can be solubilized using denaturing agents and isolated therefrom in their correct folded native conformation without removing the solubilizing denaturant. Additionally, the presence of high concentrations of denaturant usually inactivates proteases and makes an addition of protease inhibitors unnecessary.

Therefore, the current invention provides a process for the production of a correct folded protein or a salt thereof, characterized in that the protein is treated with a buffer comprising a denaturant, and the correct folded protein is separated therefrom directly, wherein the denaturant is selected from the group consisting of guanidine hydrochloride in a concentration from 3 to 7 M and urea in a concentration from 6 to 10 M.

The wording correct folded protein stands for a protein that is in the native conformation and/or shows a biological activity like the enzymatic activity or the binding property of the native protein.

The inventive process is applicable to any protein or protein fragment that has to be folded into a correct folded conformation and that establishes an equilibrium between a not correct and a correct folded conformation in presence of the denaturant. This is usually the case for proteins that are not irreversibly denatured by the denaturant. The ability of a protein to establish said equilibrium can easily be monitored by standard methods that provide information on the folding of proteins in solution like NMR or circular dicroism.

The protein to be refolded by the inventive process may be from almost any source, a special pretreatment is not necessary but not excluded. For example, a recombinant protein that is stored in the producing host in form of inclusion bodies can be refolded by simply separating the inclusion bodies from the rest of the cell debris, solubilizing the proteins of the inclusion bodies with denaturant and isolating the correct folded protein therefrom. In case the protein is not stored in form of inclusion bodies it is possible to enrich the protein only to some extend using, e.g., a precipitation step, solubilize the protein with the denaturant and isolate the pure and correct folded protein therefrom. For recombinant proteins or natural proteins that are not in a correct folded conformation after isolation it is possible to solubilize these protein after isolation in the denaturant and isolate the correct folded fraction therefrom.

Examples for suitable proteins are hirudin, epidermal growth factor, potato carboxypeptidase inhibitor (PCI) and bovine pancreatic trypsin inhibitor (BPTI), IGF-1, C5aantagonist, TGF-ss. Especially preferred proteins are himdin and epidermal growth factor (EGF).

The term hiwdin as used in this invention is intended to embrace all desulfatohirudin compounds described in literature or obtainable from a transformed microorganism strain containing DNA which codes for a desulfatohirudin or a derivative thereof. Such hinudins are, for example, desulfatohirudin derivatives HV1, HV2 and HV3 (PA), as well as other hiwdin proteins as described e.g. by M. Scharf et al. (FEBS Lett., 255 (1989), 105-110) and EP-A-347376. It is to be understood that hiwdin derivatives or shorter fragments having hiwdin activity are also covered by the term "hirudin". Such fragments and derivatives are, for example, C-terminally shortened desulfatohirudins.

Since not correct folded (scrambled) species and correct folded (native) protein exist in equilibrium under denaturant, one may design conditions to tip the equilibrium in favor of the native conformation. One possible approach is to remove the native species continuously during the folding. Another strategy is simply to recycle the scrambled species. This can be achieved by isolating the scrambled species and allowing them to reequilibrate in the denaturant in order to generate the native protein. Knowing the equilibrium constant under selected denaturing conditions, one can calculate the total yield of native protein using the following formula,
Yield = A. . + B +B+B2+ ... +bun) where n represents the times of recycling, A and B represent percentages of native protein and scrambled species presented in equilibrium. A and B, both are smaller than 1, can be readily derived from the equilibrium constant.

Another or additional possibility to shift the equilibrium towards the correct folded conformation is the addition of substances that promote correct folding like metal salts that stabilize the correct conformation or removing the native species using solid bound ligand that binds specifically to the native structure.

In a preferred embodiment of the invention, the correct folded and the not correct folded protein are separated continuously or discontinuously.

All denaturants that allow the solubilized protein to establish an equilibrium between not correct folded and correct

folded conformation can be used in the process according to the invention.

The concentration of guanidine hydrochloride is preferably from 4 to 6 M and the concentration of urea is preferably 7 to 9 M.

If the protein to be folded comprises in the correct folded conformation one or more disulfide bonds the addition of a reducing agent or a redox system is recommended.

Advantageously, the process is carried out in presence of a reducing agent with a redoxpotential from -0.20 to -0.30 such as glutathione, cysteine or 5-mercaptoethanol which is preferred. The concentration of the reducing agent is preferably from 0.05 to 1 mM and more preferably from 0.1 to 0.5 mM.

The denaturation buffer or the buffer to that the correct folded protein is isolated may also contain additional compound that promote folding or prevent undesired side reactions.

Examples are further denaturants like SDS and Triton' or metal ions, further reducing agents, oxidizing agents, complexing agents like EDTA or co-enzymes.

The correct folded protein may be separated from the not corrected folded protein by any process that is able to distinguish said two forms. Said processes are, e.g., based on a difference in mobility, shape, reactivity or binding properties. Examples for suitable processes are antibody based, membrane based, electrophoretic or chromatographic separations like gel electrophoresis, gel filtration, thin layer chromatography (TLC), HPLC, affinity chromatography or separation via a selective membrane. In a preferred embodiment of the invention the correct folded protein is separated by HPLC, TLC or affinity chromatography.

In case the correct folded protein is separated discontinuously the conditions that are necessary to establish said equilibrium have to be adapted again, e.g., by concentration or dilution of the remaining solution. It is for example possible to isolate the correct and the not-correct folded protein separately, e.g. with a HPLC column, concentrate the fraction containing the not correct folded protein and solubilize it once more under denaturating conditions. If the separation process is carried out continuously the concentrations of the essential components can be monitored, e.g. using spectroscopic means, and continuously adapted.

The folding reactions is carried out preferably at a temperature that promotes the establishment of an equilibrium and does not irreversibly denature the protein. Therefore, the applied temperature mainly depends on the stability of the protein and the separation procedure for the correct folded protein. For example, certain proteins of thermophile microorganisms are stable at 60"C and above while proteins that originate from not thermophilic microorganisms might sometimes enter irreversible modifications at 40"C or below.

Brief descriPtion of the drawings
In the following experimental part various embodiments of the present invention are described with reference to the accompanying drawings in which:
Fig. 1 is a HPLC protocol of the hirudin core domain (HirlA9) in presence of 5 M guanidine hydrochloride and 0.25 mM ss-mercaptoethanol.

Fig. 2 is a HPLC protocol of epidermal growth factor in presence of 3 M guanidine hydrochloride and 0.25 mM '3-mercaptoethanol.

EXPERIMENTAL PROCEDURES
Example 1: Production and isolation of the hiwdin core domain (Hir'49)
Recombinant desulfated hirudin was is isolated from Saccharomyces cerevisiae as described in Meyhack et al. (Thromb. Res. Suppl. VII (1987), 33). The isolated desulfatohirudin is dissolved in 50mM ammoniumbicarbonate buffer pH 8.0 at a concentration of 5 mg/ml and digested with chymotrypsin (0.25 mg/ml) at room temperature for 16 h. The digestion is terminated by addition of trifluoroacetic acid to a final concentration of 0.8 % and the core domain (Hir149) is isolated by HPLC.

Condition for HPLC:
Column: Vydac C-18
Solvent A: 0.1 % trifluoroacetic acid in water
Solvent B: 0.1 % trifluoroacetic acid in acetonitrile
Gradient: 10 % B to 48 % Bin 30 min at 23"C

Retention time for Hir'49: 14.3 min
Example 2: Denaturation of the hiwdin core domain (Hir'49)
The starting material for the folding experiments, fully reduced / denatured core domain of hiwdin [Rj, is prepared by the following method:
The hinrdin core domain from examplel (2 mg/ml) is dissolved in Tris-HCl buffer (0.5 M, pH 8.5) containing 5 M of guanidine chloride (GdmCl) and 30mM of dithiothreitol.

Reduction and denaturation are carried out at 23"C for 90 min. To initiate the folding, the sample is passed through a PD-10* column (Pharmacia) equilibrated in 0.1 M Tris-HCl buffer (pH 8.5). Desalting takes about 1-2 min and the sample is immediately used in the folding experiments.

Example 3: Foldinq of hirudin in the presence of puanidine hvdrochlolide
The samples are diluted to a final protein concentration of 1 mg/ml; containing 0.1 M Tris
HCl buffer (pH 8.5), 5 M guanidine hydrochloride and 0.25 mM ss-mercaptoethanol. After 24 h incubation at room temperature, the native hinrdin is separated from scrambled himdin via HPLC.

Condition for HPLC:
Column: Vydac C-18
Solvent A: 0.1 % trifluoroacetic acid in water
Solvent B: 0.1 % trifluoroacetic acid in acetonitrile
Gradient: 14 % B to 32 % B in 50 min at 23 C
Retention time for Hit1-49: 23 min
The HPLC-protocol is given in Figure 1.

From the integration of the HPLC-protocol values the amount of native hiwdin is calculated to 60% # 5% and the Keqto 0.67 j0.15.

The fraction containing the scrambled hirudin is lyophilized and dissolved in 0.1 M Tris-HCl buffer (pH 8.5) containing 5 M guanidine hydrochloride and 0.25 mM ss-mercaptoethanol to a final protein concentration of 1 mg/ml. After 24 h incubation, the native hiwdin is separated from scrambled hiwdin via HPLC as described above. Lyophilization and renaturation is carried out for a third time as described above.

All fractions containing the native hirudin are combined and the total recovery of native hiwdin sums up to 97 % after three cycles.

The activity of the recovered hiwdin is proved by the ability to inhibit human o-thrombin from digesting Chromozym (Boehringer Mannheim). The reaction is carried at 22 C in 67 mM Tris-HCl buffer (pH 8.0) containing 133 mM NaCl and 0.13 % polyethylene glycol 6000. The rate of digestion was followed at 405 nm for a period of 2 min. The concentration of substrate is 200 mM. The concentration of thrombin is adjusted in between 2.5 and 25 nM.

For further structural analysis the recovered protein is carboxymethylated with 0.2 M iodoacetic acid in Tris-HC1 buffer (0.5 M, pH 8.5) for 30 min and desalted through a PD-i 00 column equilibrated with ammonium bicarbonate solution (50 mM, pH 8.0). The disulfide contents is determined by amino acid analysis (Chang and Knecht, Anal. Biochem. (1991), 197, 52-58) and mass spectrometry (Chatrenet and Chang, J. Biol. Chem. (1992), 267, 3038-3043).

Example 4: Foldinq of himdin in the presence of urea
The renaturation is carried out as described in example 3 with the sole difference that 8 M urea is used instead of 5 M guanidine hydrochloride.

From the integration of the HPLC-protocol values the amount of native hiwdin is calculated to 90 % + 5 % and the Key to 0.11 + 0.06.

After two cycles, the total recovery of native hirudin is 99 %.

Example 5: Denaturation of enidermal growth factor
Epidermal growth factor (EGF) is provided by Protein Institute Inc. (Broomall, USA) and is denatured as described for hirudin in example 2.

Example 6: Folding of EGF in the presence of guanidine hydrochloride
The renaturation is carried out as described in example 3 with the sole difference that EGF is used instead of hiwdin

and 3 M guanidine hydrochloride is used instead of 5 M guanidine hydrochloride.

The HPLC-protocol is given in Figure 2.

From the integration of the HPLC-protocol values the amount of native EGF is calculated to 89% ~ 5 % and the K,to 0.12 f 0.07.

After two cycles, the total recovery of native EGF is 99%.

The disulfide content is determined as described in example3.

Claims

Claims:

1. A process for the production of a correct folded protein or a salt thereof, characterized in that the protein is treated with a buffer comprising a denaturant, and the correct folded protein is separated therefrom directly, wherein the denaturant is selected from the group consisting of guanidine hydrochloride in a concentration from 3 to 7 M and urea in a concentration from 6 to 10 M.

2. A process according to claim 1, wherein the correct folded protein is separated continuously or discontinuously.

3. A process according to claim 1, wherein the correct folded protein is separated discontinuously.

4. A process according to claim 1, wherein the protein is hirudin or epidermal growth factor.

5. A process according to claim 1, wherein the concentration of guanidine hydrochloride is from 4 to 6 M.

6. A process according to claim 1. wherein the concentration of urea is 7 to 9 M.

7. A process according to claim 1, wherein a reducing agent is present.

8. A process according to claim 7, wherein the reducing agent has a redoxpotential from 0.20 to -0.30.

9. A process according to claim 7, wherein the reducing agent is ss-mercaptoethanol.

10.A process according to claim 7, wherein the concentration of the reducing agent is from 0.05 to 1 mM.

11.A process according to claim 7, wherein the concentration of the reducing agent is from 0.1 to 0.5 mM.

12.A process according to claim 1, wherein the correct folded protein is separated by antibody based, membrane based, electrophoretic or chromatographic separations.

13.A process according to claim 1, wherein the correct folded protein is separated by HPLC, TLC or affinity chromatography.
